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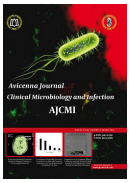

Original Article

The Role of Efflux Pumps in the Antibiotic Resistance of *Campylobacter* spp. Isolated From Domestic Animals and Poultry

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Abstract

Background: Recently, the rate of antibiotic resistance of *Campylobacter* has been reported to be increasing and the mechanism of this resistance has been reported to be related to the activity of efflux pumps. The purpose of this study was to isolate *Campylobacter* strains from domestic animals such as poultry and cows and evaluate the role of efflux pumps in antibiotic resistance property of them.

Methods: A total of 300 fecal samples were collected from poultry and cows and subjected to isolation of *Campylobacter* by preT-KB method. The isolates were identified and confirmed by phenotypic and genotypic methods and their antibiotic susceptibility was evaluated using the disk diffusion method. Efflux pump activity in the isolates was assessed by EtBr-agar cartwheel method and the presence of efflux pump *cme*ABC was evaluated in all isolates. Finally, the correlation between efflux pump activity and antibiotic resistance was evaluated in the isolates using inhibition of efflux pump activity of Phe-Arg β-naphthylamide.

Results: Of all samples, 10 (3.3%) *Campylobacter* strains were isolated. Seven (70%) and three (30%) strains were isolated from poultry and cows, respectively. Of all isolates, 9 belonged to *Campylobacter jejuni* and 1 belonged to *Campylobacter coli*. The isolates were resistant to three antibiotics, namely Ciprofloxacin, Ceftriaxone, and Cefotaxime. Efflux pump activity was observed in all isolates; however, *cme*ABC genes were not present in all of them. In addition, resistance to Erythromycin and Ciprofloxacin was associated with efflux pump activity.

Conclusions: All *Campylobacter* isolates in the current study showed antibiotic resistance and the activity of efflux pumps could induce antibiotic resistance and decrease the antibacterial activity of many drug families in *Campylobacter*. In addition, the activity of efflux pumps can be considered a mechanism of antibiotic resistance and elimination of this activity might increase the effectiveness of antibiotics.

Keywords: *Campylobacter*, Antibiotic resistance, Efflux pumps

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Background

Campylobactereaceae include the genera *Campylobacter*, *Achromobacter*, and *Sulfurosprillium*. 23SrRNA analysis opined that this family belongs to delta/epsilon of proteobacteria (1). *Campylobacter* species are gram-negative, motile, non-spore-forming, and spiral-shaped organisms. They are microaerophilic, nonsaccharolytic, nonproteolytic, and nonlipolytic so they do not ferment or oxidize carbohydrates. These bacteria live in the gastrointestinal tracts of birds and warm blooded animals. *Campylobacter* can grow on artificial media such as Kapadnis-Baseri (KB) medium under microaerophilic conditions. Recently, *Campylobacter* has been introduced as an important food poisoning agent and an emerging pathogen. *Campylobacter* genus is divided into two groups based on the production of catalase. *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter lari* are catalase-positive human pathogens. However, the other species are considered catalase-negative or weak *Campylobacter* and are almost non-pathogenic (2). *Campylobacter jejuni* is the most important cause of diarrhea. Invasive factors of this bacterium are colonization, flagella, lipopolysaccharide (LPS) , and production of toxins and antigens (3). The main transmission routes of *Campylobacter* include drinking of contaminated water and consumption of meat and dairy products (1). Nowadays, higher frequencies of antibiotic prescriptions culminated in the emergence of antibiotic-resistant *Campylobacter*. Moreover, antibiotic therapy in used in immunocompromised or elderly people to prevent infections. In this regard, HIV and diabetes mellitus patients are at risk of *Campylobacter* dissemination; therefore, the high rate of antibiotic prescription is needed for their treatment (4). In addition, plasmid-mediated antibiotic resistance in some *Campylobacter* isolates could

increase the rate of *Campylobacter* infections among the human population (5). Gene modification and the production of drug-inactivating enzymes are the most important mechanisms of antibiotic resistance. On the other hand, the activity of efflux pumps could induce antibiotic resistance in many bacteria. Hydrolysis of ATP by these pumps reduces the antibiotic concentrations in the bacteria and subsequently increases the rate of antibiotic-resistant bacteria (6). Therefore, the present study was undertaken to investigate the role of efflux pumps (CmeABC) in inducing antibiotic resistance in *Campylobacter* isolates.

Materials and Methods

Sample Collection and Isolation of Campylobacter spp.

In this cross-sectional study, 300 fecal samples were collected from cows and poultry in different farms in Fars, Bushehr, and Khuzestan provinces in the south of Iran. The samples were collected using sterile sticks and polyethylene bags and transferred to the laboratory within one hour of sampling. The samples were subjected to isolation of *Campylobacter* using the preT-KB method (2). In this method, *Campylobacter* was cultivated on the Mueller-Hinton agar. To perform the experiment, 1 g of fecal samples was emulsified in sterile phosphate buffered saline (pH 7.0, 0.1 M) at 10% (w/v) concentration. The suspension was centrifuged at 8500 rpm for 10 minutes. Then, the tube was kept at room temperature for 10 minutes. Afterwards, a loopful of supernatant was cultivated on the Mueller-Hinton agar and the plates were incubated at 37°C for 48 hours. The isolates were phenotypically identified using Gram staining, glucose, oxidase, and catalase tests (2).

Confirmation of Campylobacter Isolates

Ten suspected *Campylobacter* strains were subjected to 16S rRNA gene sequencing. DNA extraction was performed using PCR kit (CinnaGen, Iran). The purity of the extracted DNA was evaluated by a biophotometer (Eppendorf, Germany) based on 260 and 280 nm wavelengths ratio. PCR mixture of each reaction contained master mix (CinnaGen, Iran) and forward and reverse primers of univrsal16S rRNA. Thermal program included 95°C for 4 minutes, followed by 32 cycles of 95°C for 5 minutes, 94°C for 35 seconds, 56°C for 40 seconds, and 72°C for 50 seconds with a final extension at 72°C for 5 minutes and storage at 4°C (Table 1) (7).

All PCR products were run on 1% (w/v) agarose gel along with 5 µL of 100 bp DNA ladder. The pure 16S rRNA PCR products were sent to Macrogen in South Korea (http://www.macrogen.com/) for DNA sequencing. Then, BLAST analysis was done (http://www.ncbi.nlm.nih.gov/BLAST/). It means similarity of the 16S rDNA sequence of all isolates was evaluated against corresponding nucleotide sequences retrieved from GenBank.

Antibiotic Susceptibility

Antimicrobial susceptibility of *Campylobacter* spp. isolates was assessed by the disc diffusion method. To perform the test, each isolate was inoculated in trypticase soy broth and incubated at 37°C for 48 hours under microaerobic conditions. Then, 0.1 mL of the suspension (0.5 McFarland standard tubes (1.5×10^8 cells mL^{-1})) was picked and streaked on the Mueller-Hinton agar. Afterwards, the antibiotic discs, including ampicillin (10 µg), ceftriaxone (30 µg), cefotaxime (30 µg), gentamicin (10 µg), erythromycin (15 µg), and ciprofloxacin (5 µg) (Patanteb, Iran), were placed on the plates and incubated at 37°C. After 48-72 hours, the inhibition zone of each disk was measured, and based on Clinical and Laboratory Standards Institute (CLSI) 2018 guidelines, the susceptibility of the isolates was analyzed by WHONET 5.6 and recorded (2).

Table 1. Primers Used in the Present Study

Primers	Sequence	Length	Reference
Camp.F[a]	5´-GGATGACACTTTTCGGAGC-3´	19	7
Camp.R[a]	5´-CATTGTAGCACGTGTGTC-3´	18	7
cmeA.F [b]	5´-TGGGGTATTCATTGTTTTGGTAG-3´	23	9
cmeA.R [b]	5´-ATACAAATGCCGCCTCAACC-3´	20	9
cmeB.F [b]	5´-CCAAATACCGCAAAAGGTACAG -3´	22	9
cmeB.R [b]	5´-CCTCTGTATTTAGCGCAGGAG -3´	21	9
cmeC.F [b]	5´-GCCAATTTTGACGTGCCTCT -3´	20	9
cmeC.R [b]	5´-GCGGTAGTCGTGCAAAAACA -3´	20	9

[a]16S rDNA primers; [b] Efflux pump primers

Evaluation of Efflux Pump activity in Campylobacter Isolates

Detection of efflux pump activity was done by EtBr cartwheel method. To perform the test, Ethidium Bromide was serially diluted with sterile distilled water (1/2, 1/4, and 1/8). Then, 10 mL of each solution was added into 200 mL of melted sterile nutrient agar and plated (the process was done under laminar flow hood). Then, *Campylobacter* isolates were streaked on the solid agar medium and incubated at 37°C for 48-72 hours. The observation of visualized transillumination under UVTEC for each isolate was considered the inactivity of efflux pump and vice versa was considered the efflux pump activity (8).

Detection of Efflux Pump Genes

Genotypic detection of the efflux pump genes was performed using specific primers shown in Table 1 (9). The experiment was carried out as mentioned above except for primers and PCR temperatures (denaturation: 94 °C; annealing: *cme*A: 56°C, *cme*B:56°C, and *cme*C: 58°C; extension : 72°C).

Correlation Between Antibiotic Resistance and efflux Pump Activity

Phe-Arg β-naphthylamide is a special compound for eliminating the efflux pump activity. Hence, this compound was used to achieve information concerning the correlation between antibiotic resistance and efflux pump activity. To perform the test, two flasks containing

200 mL of sterile melted Mueller Hinton agar mixed with 1 mL of Phe-Arg β-naphthylamide (concentration of 0.05 mg in 30 mL D.W). Then, the isolates were streaked on the medium and ampicillin (10 µg), ceftriaxone (30 µg), cefotaxime (30 µg), gentamicin (10 µg), erythromycin (15 µg), and ciprofloxacin (5 µg) discs were placed (distance of each disk from another was 24 mm) on the medium. Afterwards, the plates were incubated at 37°C. After 48-72 hours, the inhibition zone of each disk was measured, and based on CLSI, 2018 guidelines, the susceptibility of the isolates was evaluated and recorded (10).

Statistical Analysis

Paired student's *t* test was used to determine the correlation between antibiotic resistance and efflux pump activity. *P* values of < 0.05 were considered significant.

Results

Isolation and Identification of Campylobacter spp.

Of all samples, 10 (3.3%) *Campylobacter* strains were isolated. Seven (70%) and 3 (30%) strains were isolated from poultry and cows, respectively. As seen in Table 2, 9 strains belonged to *Campylobacter jejuni* and 1 strain belonged to *Campylobacter coli*. Gel electrophoresis of 16S rDNA PCR products is shown in Figure 1. As seen in this figure, all *Campylobacter* 16SrDNA genes had a DNA fragment of 1232 bp.

Antibiotic Susceptibility

The results obtained indicated that all strains were resistant to ciprofloxacin, ceftriaxone, and cefixime. However, 70% and 20% of the isolates were resistant to erythromycin, ampicillin, and gentamicin, respectively (Figure 1).

Table 2. Confirmation of *Campylobacter* Isolates

Campylobacter Strains	Genotypic Confirmation	Accession Number
C1	*Campylobacter jejuni* strain ZJB020	CP040613.1
C2	*Campylobacter jejuni* strain AR-0419	CP044162.1
P1	*Campylobacter jejuni* strain AR-0419	CP044162.1
P2	*Campylobacter jejuni* strain AR-0413	CP044171.1
P3	*Campylobacter jejuni* strain NCTC13257	LR134502.1
P4	*Campylobacter jejuni* strain NCTC13261	LR134500.1
P5	*Campylobacter jejuni* strain NCTC13266	LR134496.1
P6	*Campylobacter jejuni* strain CFSAN032806	CP045789.1
C3	*Campylobacter coli* RM4661	CP007181.1

Efflux Pump Activity in Campylobacter Isolates

The results obtained indicated that all the isolates showed efflux pump activity. In other words, transillumination was not observed in *Campylobacter* colonies. Furthermore, PCR products and gel electrophoresis showed the presence of *cme*A, *cme*B, and *cme*C. As seen in these figures, DNA fragments of 661, 1153, and 838 bps were seen for *cme*A, *cme*B, and *cme*C, respectively. As seen in Figure 2, *cme*A gene was absent in three strains of *Campylobacter jejuni* (lines 1, 6, and 9). Figure 3 shows the detection of *cme*B gene in all the isolates except for three strains of *Campylobacter jejuni* (columns 1, 5, and 10). Figure 3 shows the detection of *cme*C gene in all the isolates except for two strains of *Campylobacter jejuni* (columns 6 and 9).

Correlation Between Antibiotic Resistance and Efflux Pump Activity in Campylobacter Isolates

The results obtained indicated that Phe-Arg β-naphthylamide could not increase the antimicrobial activity of 4 antibiotics including ampicillin, ceftriaxone, cefotaxime, and erythromycin. However, gentamicin and ciprofloxacin showed antimicrobial activity in the presence of Phe-Arg-β-naphthylamide. Statistical analysis of data in Table 3 shows significance values for ciprofloxacin and gentamicin were less than 0.05 and the value of confidence interval for both antibiotics were less than zero. Hence, a positive correlation was found between efflux pump activity and gentamicin and ciprofloxacin resistance in *Campylobacter* isolates. However, no correlation was found between efflux pump activity and resistance to other antibiotics in *Campylobacter* isolates.

Discussion

Campylobacter infection was recognized as a zoonosis, for which several antibiotics have been prescribed (2). In the present study, *Campylobacter* was isolated from cows and poultry with a prevalence of 3.3%. The rate of *Campylobacter* isolation in the present study was lower compared to other reports, which may be due to differences in the climate and diet of animals and poultry

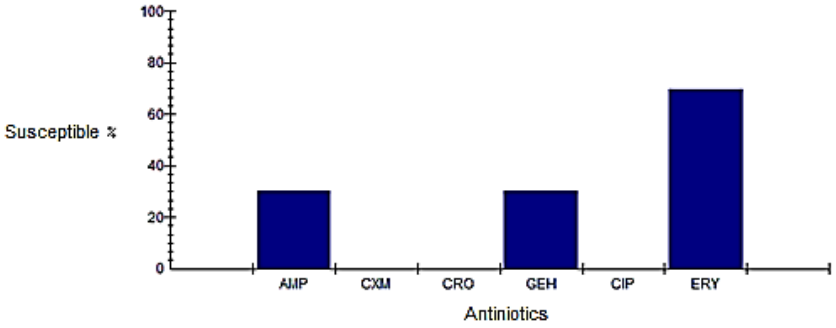

Figure 1. Antibiotic Susceptibility of *Campylobacter* Isolates. AMP: Amoxicillin, CXM: Cefotaxime, Ceftriaxone, CIP: Ciprofloxacin, GEH: Gentamicin, ERY: Erythromycin.

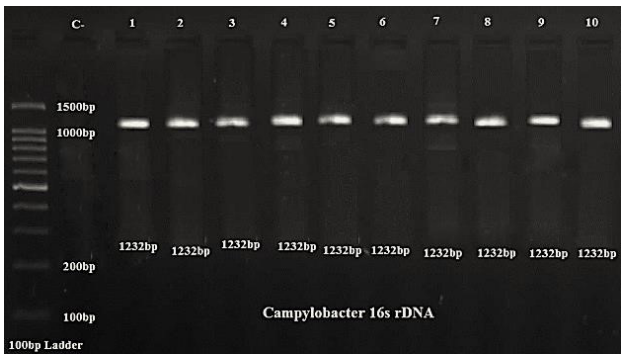

Figure 2. Agarose Gel Electrophoresis of 16S rDNA PCR Products of *Campylobacter.*

(11). In a different study, Baserisalehi et al reported that the prevalence of *Campylobacter* in poultry was relatively higher compared to camels because of their diet (1).

Recently, high consumption of antibiotics culminated in the emergence of antibiotic-resistant bacteria. In this regard, several mechanisms are responsible for developing the antibiotic-resistant bacteria (12). RNA efflux pump has an operon coded by three genes of *cme*A, *cme*B and *cme*C which are essential for *Campylobacter* colonization in the intestinal tract (13). Yao et al in 2016 reported that efflux pumps increase antibiotic resistance in *Campylobacter* (14). Several studies showed that efflux pumps can mediate resistance to norfloxacin, imipenem, ciprofloxacin,

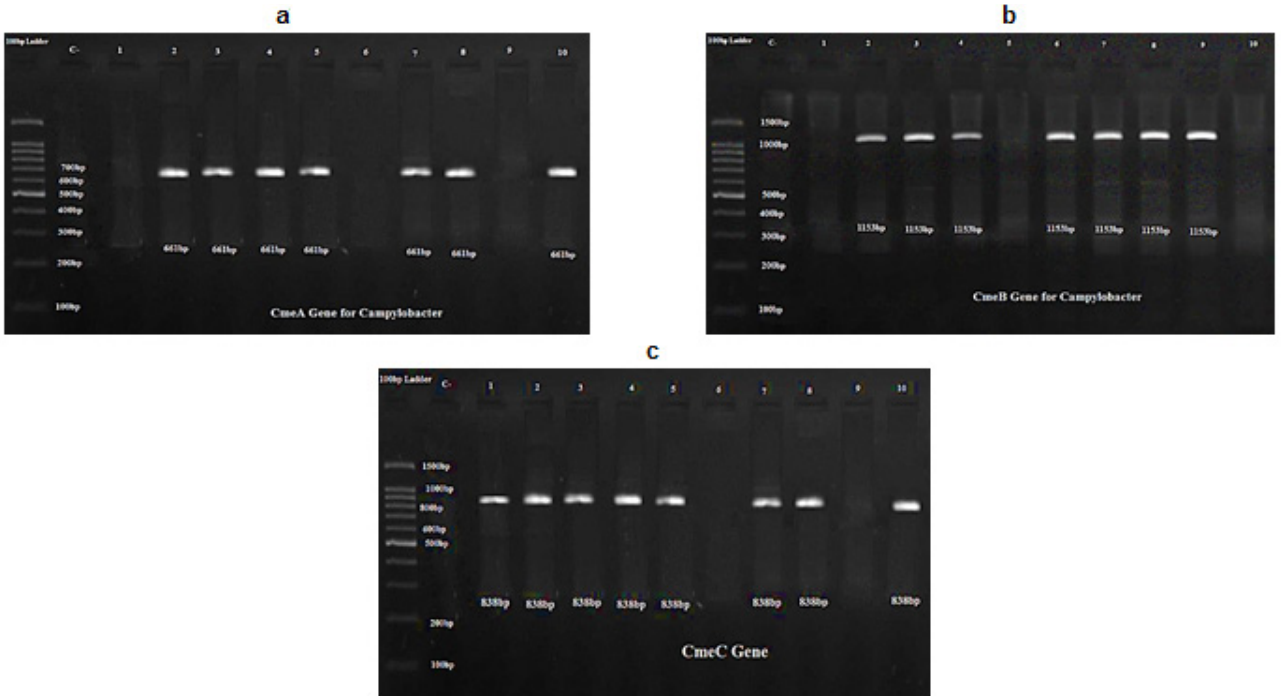

Figure 3. PCR Product of *Campylobacter* Isolates showing Detection of (a) *cmeA,* (b) *cmeB*, and (c) *cmeC* Genes.

Table 3. Statistical Analysis of Efflux Pump Activity and Antibiotic Resistance in *Campylobacter* Isolates

Antibiotics	Levene's Test for Equality of Variances		*T* test for Equality of Means						
	F	Sig.	T	df	Sig. (two-tailed)	Mean Difference	Standard Error Difference	95% CI of the difference	
								Lower	Upper
Amoxicillin	0.025	0.876	-0.331	18	0.744	-1.000	3.021	-7.346	5.346
			-0.331	17.982	0.744	-1.000	3.021	-7.347	5.347
Cefotaxime	0.112	0.741	-1.857	18	0.080	-0.600	0.323	-1.279	0.079
			-1.857	17.967	0.080	-0.600	0.323	-1.279	0.079
Ceftriaxone	0.112	0.741	-1.857	18	0.080	-0.600	0.323	-1.279	0.079
			-1.857	17.967	0.080	-0.600	0.323	-1.279	0.079
Ciprofloxacin	6.369	0.021	-24.252	18	0.000	-17.600	0.726	-19.125	-16.075
			-24.252	12.950	0.000	-17.600	0.726	-19.168	-16.032
Gentamicin	6.529	0.020	-22.204	18	0.000	-16.500	0.743	-18.061	-14.939
			-22.204	14.015	0.000	-16.500	0.743	-18.094	-14.906
Erythromycin	0.067	0.798	-0.642	18	0.529	-1.100	1.714	-4.702	2.502
			-0.642	17.932	0.529	-1.100	1.714	-4.703	2.503

erythromycin, cefotaxim, and tetracycline (15,16). Our finding verified the existence of *Campylobacter* in the intestinal tract of domestic animals and poultry in our area. In addition, the isolates were resistant to some antibiotics such as ampicillin, cephalothin, ciprofloxacin, cefotaxime, erythromycin, and gentamicin with different percentages. In some countries such as Thailand and India, 80% and 77% of *Campylobacter* isolates, respectively, were resistant to fluoroquinolones (17). Even in China, 95.8%–99% of *Campylobacter coli* isolates were resistant to ciprofloxacin (18). Resistance to gentamicin and ciprofloxacin in the *Campylobacter* isolates was related to efflux pump activity. Hence, according to this data, resistance to aminoglycoside and quinolones in *Campylobacter* isolates was mediated by efflux pumps. Several reports supported our finding, for instance, Gibreel et al showed a relationship between resistance to Macrolides in *Campylobacter* spp. and efflux pump activity (18). In addition, Bolinger and Kathariou in 2017 reported a relationship between resistance to fluoroquinolones and Macrolides and the presence of *cme*ABC genes in *Campylobacter jejuni*. They stated that the MIC of macrolide-resistant *Campylobacter* was affected by mutations in the regulatory region of *cme*ABC (13). The results of the current study indicated the activity of efflux pumps in all *Campylobacter* isolates; however, *cme*ABC genes were not found in all of them.

Conclusions

Nowadays, several antibiotics are used for the treatment of *Campylobacter* disease. Hence, the rates of resistance to antibiotics among *Campylobacter isolates* are increasing. In this regard, our finding showed a high prevalence of antibiotic-resistant *Campylobacter* and efflux pumps activity was introduced as a major mechanism of antibiotic resistance. In addition, plasmid-mediated antibiotic resistance in some *Campylobacter isolates* could increase the rate of *Campylobacter* infections among the human population.

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Authors' Contribution

MBS designed the investigation and PM did all experimental procedures.

Conflict of Interests

The authors declared no conflict of interests.

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